Reference: *Biol. Bull.* **188**: 313–328. (June, 1995)

Experimental and Histological Studies of Four Life-History Stages of the Eastern Oyster, *Crassostrea virginica,* Exposed to a Cultured Strain of the Dinoflagellate *Prorocentrum minimum*

GARY H. WIKFORS[1] AND ROXANNA M. SMOLOWITZ[2]

[1]*National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford, Connecticut 06460; and* [2]*Laboratory for Marine Animal Health, University of Pennsylvania, Marine Biological Laboratory, Woods Hole, Massachusetts 02543*

Abstract. Effects of the dinoflagellate *Prorocentrum minimum* (strain EXUV) upon four life-history stages of the eastern oyster—embryos, feeding larvae, newly set spat, and juveniles—were investigated in laboratory exposure studies. Embryonic development was not affected significantly by living, heat-killed, or sonicated cells, or by growth-medium extracts from *P. minimum* cultures. Feeding larvae, however, showed poor growth and poor development of the digestive system when fed *P. minimum,* as compared with larvae fed *Isochrysis* sp. (strain T-ISO). Growth of larvae fed mixed *P. minimum* + *Isochrysis* diets was intermediate. Larvae and newly set spat that had been fed a diet of ⅓ *P. minimum* + ⅔ *Isochrysis* exhibited distinctive changes in digestive-system anatomy. Spat showed an abnormal accumulation of lipid in the stomach epithelium. Absorptive cells in the digestive glands of both larvae and spat contained accumulation bodies, often with a laminated, fibrous appearance in preparations for transmission electron microscopy. These accumulation bodies were PAS (periodic acid-Schiff) positive and may correspond to autolysosomal bodies within *P. minimum* cells. Juvenile oysters developed the ability to digest *P. minimum,* but only after a refractory period of about 2 weeks, during which most *P. minimum* was filtered but rejected as pseudofeces. The linking of accumulation bodies within absorptive cells of oyster digestive diverticula with dinoflagellate autolysosomal bodies suggests a mechanism by which some dinoflagellates interfere with feeding in phytoplankton grazers.

Received 1 September 1994; accepted 21 February 1995.

Introduction

Harmful algal blooms are viewed as an increasing threat to utilization of living marine resources (Hallegraeff, 1993). Studies of interactions between dinoflagellates and molluscan shellfish have focused mainly on the accumulation of mammalian neurotoxins by molluscs feeding on toxic dinoflagellates (Sakamoto *et al.,* 1987). More recently, the detrimental effects of toxic dinoflagellates, as well as the effects of some algae that produce no known mammalian toxins, upon the shellfish themselves have been identified (Shumway, 1990). Responses ranging from reduced filtering to increased metabolic rates, paralysis, and mortality have been noted for molluscs feeding upon natural populations of dinoflagellates or cultured strains (Gainey and Shumway, 1988). It has, in fact, been suggested that detrimental effects upon shellfish by a number of phytoplankton taxa may be undetected rather than uncommon (Parry *et al.,* 1989).

Among dinoflagellate taxa implicated in toxic effects are members of the genus *Prorocentrum.* Two benthic *Prorocentrum* species, *P. lima* and *P. mexicanum,* produce ciguatoxin, which accumulates in tropical finfish food chains and renders apex predators unfit for human consumption (Steidinger, 1983). There are historical accounts of *P. minimum*—and its taxonomic equivalents *P. mariae-lebouriae, Exuviaella mariae-Lebouriae,* and *P. triangulatum* (Dodge, 1982)—causing toxic effects in both shellfish and human consumers of shellfish harvested from *P. minimum* bloom water (Shumway, 1990). One strain of *P. minimum* that is cultured in a number of laboratories, clone EXUV (CCMP1329), yielded negative results

in previous (Schmidt and Loeblich, 1979) mouse bioassays for water-soluble mammalian toxins (PSP) and in assays carried out during the present study (S. E. Shumway, pers. comm.). Despite the negative finding for mammalian toxin in this strain, we demonstrated previously that EXUV cultured in our laboratory did not support the growth of juvenile northern quahogs, *Mercenaria mercenaria*, and was acutely toxic to juvenile bay scallops, *Argopecten irradians* (Wikfors and Smolowitz, 1993). Complete mortality of scallops exposed to the EXUV strain occurred within 1–4 weeks of first exposure. Affected scallops had severe attenuation of epithelial cells associated with absorptive-cell necrosis and sloughing of cells into central lumens. Large melanized hemocyte clots were present throughout the open vascular system; these histologic observations are strong evidence for the presence of a molluscan enterotoxin in EXUV.

Because *P. minimum* is a normal component of the summertime flora in the coastal waters of the northeastern United States (Marshall, 1980; Sellner *et al.*, 1993; Wikfors, in prep.), it seemed possible that native oysters, which spawn during summer, could be exposed to this dinoflagellate at any stage of their life history. Timing, density, and geographic extent of *Prorocentrum* populations could play some role in the recruitment success of oysters if there are detrimental effects at particular life-history stages. Therefore, the present study was undertaken to expose oysters at four life-history stages—prefeeding embryos, feeding larvae, newly set spat, and juveniles—to cultured *P. minimum*, both alone and in combination with a known "good-food" alga, *Isochrysis* sp. (strain T-ISO). Survival, development, growth, and histologic condition were evaluated and compared with those of starved oysters and oysters fed T-ISO alone—an algal diet known to support normal growth and development (Wikfors, unpub.).

Materials and Methods

Phytoplankton culture

The EXUV strain of *P. minimum* (CCMP1329) was obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton; *Isochrysis* sp. strain T-ISO was in the Milford Microalgal Culture Collection. Both axenic algal strains were cultured in enriched natural seawater medium, E formulation (Ukeles, 1973), in 16-l carboy assemblies housed in a temperature-controlled (20°C) room with constant illumination of 500–600 μE m^{-2} s^{-1} from cool-white fluorescent lamps. Cultures were managed semi-continuously, with daily harvests of sufficient volumes to satisfy experimental regimes and weekly replacement of harvested volumes with sterilized medium. Culture densities were determined as packed-cell volumes by centrifugation in specially modified Hopkins tubes (Ukeles, 1973), and daily feeding regimes were specified according to packed-cell volume (specified for larvae or postset below). Cell counts were made in an Improved Neubauer hemocytometer.

Oyster rearing

Broodstock oysters from the Milford Laboratory were spawned by warm-water stimulation (Loosanoff and Davis, 1963); 5 females and 8 males contributed gametes to the cohort used in experiments. All oyster rearing was in Milford Harbor seawater (27–28‰ salinity) that was temperature-adjusted to 25°C, filtered to 0.5 μm, and passed through a flow-through ultraviolet sterilizer and an activated-carbon organic-removal cartridge. For the experiment with embryos, fertilized eggs were distributed into 1-l polypropylene beakers at a concentration of 20 ml^{-1}. Embryos were exposed to experimental treatments (see below) within 2 h of fertilization. Remaining embryos were incubated in seawater treated as above for 48 h before experiments were begun with feeding larvae. Subsamples of 48-h larvae were collected on a 36-μm-mesh monofilament nylon screen and distributed into 1-l beakers at a concentration of 20 ml^{-1}. Larvae not used in feeding experiments were reared on a diet of T-ISO and *Pavlova lutheri* (Milford strain MONO) through metamorphosis and then grown on natural phytoplankton amended with cultured algae (mostly *Tetraselmis maculata* strain TTM and *Pavlova gyrans* strain 93, with small amounts of diatoms and other flagellates). Four days before use in juvenile feeding studies, oysters were placed in filtered seawater to empty the digestive systems.

Experimental design

Oyster embryos were exposed to a variety of live and killed cells and extracts from *P. minimum* and the control alga, T-ISO, shortly after fertilization of eggs. Live algal cultures were added, along with the culture medium in which they were grown, as aliquots providing the number of cells given as a first feeding according to the regime of Rhodes and Landers (1973); *i.e.*, 0.005 ml packed cells l^{-1} larval culture containing 20 larvae ml^{-1}; for T-ISO, this was 60×10^6 cells l^{-1}, and for EXUV, 3.9×10^6 cells l^{-1}. Equal amounts of both algal strains were subjected to the following treatments before being added to embryo suspensions: (1) removal of cells by filtration to 0.22 μm, (2) sonication for 5 min at 250 W with 50/50 timed pulse, or (3) heating to 80°C for 30 min. In addition to the above treatments, live and sonicated EXUV culture, and medium from EXUV culture, were added at 5 times standard volume. Seawater-only and sterile algal medium controls were included as well. All treatments were in triplicate. Embryos were incubated on a temperature-controlled water table (25°C) for 48 h, and then evaluated microscopically for effects upon survival and development.

Counts of live-normal, live-abnormal, dead-normal, and dead-abnormal larvae were made for all treatments.

Larval oysters, which had been permitted to develop for 48 h in filtered seawater, were distributed into 1-l beakers for larval feeding experiments, also incubated on the 25°C water table. Larval populations in triplicate beakers were given the following daily feeding regimes: (1) EXUV, (2) ⅓ EXUV + ⅔ T-ISO, (3) ⅔ EXUV + ⅓ T-ISO, (4) T-ISO, and (5) unfed. Daily rations were adjusted according to packed-cell volume, following the progressively increasing schedule of Rhodes and Landers (1973). Concentrations (cells per liter) of T-ISO and EXUV alone on the first day of feeding are given in the previous paragraph; cells in mixed diets and in increasing rations can be calculated from these. Three days each week, larvae were collected on a 75-μm monofilament nylon screen, washed with a pressurized flow of seawater, and resuspended in newly filtered seawater. At this time, subsamples of larvae were counted on a Sedgewick-Rafter slide under a dissecting microscope and measured (shell length) on a compound video-microscope using image-analysis PC-computer software. *In vivo* fluorescence, pH, and salinity of spent larval-culture water were measured every 3 days as well; water for these samples was poured gently through a 36-mesh nylon screen to minimize the effect of fecal material upon the fluorescence measurements. Subsamples of larvae were taken periodically and fixed for histological examination; results for samples taken 8 days after spawning are reported. Larvae fed T-ISO, EXUV, and the ⅓ EXUV + ⅔ T-ISO diet were observed live, 60 min after feeding on day 14, under epifluorescence microscopy (Babinchak and Ukeles, 1979) to determine if larvae were ingesting and digesting algal cells.

In cultures that produced pedi-veligers, whole oyster shells were provided as setting substrate, and setting success was determined by counting spat on shell surfaces. Newly set spat continued to receive the diet provided to them as larvae. Spat were sampled for histological examination 5 days after setting.

The subpopulation of oysters from the same cohort, which was reared separately to a mean size of 8.3 mm shell length, was divided into groups of between 15 and 25 and placed in molluscan rearing chambers designed for conducting nutritional studies with young shellfish (Ukeles and Wikfors, 1982). Seawater supplying the chambers was filtered to 0.5 μm, passed through an ultraviolet sterilizer, and temperature-adjusted to 25°C in a head tank. Seawater then was distributed to chambers at a flow rate of 600 ml min^{-1} through a flow-adjustable manifold. The same unialgal and mixed diets were fed to juvenile oysters as were fed to larvae. Daily rations were 0.006 ml packed cells $oyster^{-1}$ d^{-1}, and percentage mixes were made by packed-cell volume, not cell number. A full ration of T-ISO consisted of 143×10^6 cells per oyster, and a full ration of EXUV was 9.27×10^6 cells per oyster; cell numbers in percentage diets can be calculated from these values. Seawater flow through the chambers was continuous, except for the 4 h after feeding, during which flow was stopped. Fluorometer measurements showed that all algal food suspensions were cleared within 2 h of feeding. Each week, for 6 weeks, oysters were removed from chambers and cleaned with a pressurized spray of seawater; with the aid of image-analysis software on an IBM-compatible microcomputer, shell lengths were measured from video images.

Histology

Subsamples of 48-h larvae, feeding larvae, and newly set spat were processed in plastic (Embed 812, Electron Microscopic Sciences). Briefly, samples fixed in 1% glutaraldehyde/4% formalin in 28‰ NaCl (1G4F) (Howard and Smith, 1983) were rinsed with phosphate buffer, secondarily fixed in 1% osmium, and hydrated to unbuffered water. Oysters were decalcified in 2% ascorbic acid in 0.9% sodium chloride at pH 2.3 for 15–20 h, followed by a rinse of unbuffered water. Samples were concentrated before removal of each solution by centrifuging at $1300 \times g$ for 3 min, followed by removal of the supernatant. Oysters then were embedded in 3% agarose (aqueous). Enbloc staining was done in 2% uranyl acetate (aqueous) overnight. Staining was followed by infiltration, embedment in Embed 812, and polymerization by standard methods. Thick sections cut from the plastic-embedded samples were stained with 1% methylene blue in 1% borax (sodium borate) and 1% azure II. Ultrathin sections were stained with 2% uranyl acetate and counter-stained with Reynolds lead-citrate. Thick sections were examined and photographed using an Olympus BH-2 photomicroscope. Thin sections were examined on a Zeiss 10 transmission electron microscope.

Juvenile oysters were fixed *in situ* by placing the oyster shells with attached juveniles into large bottles containing 1G4F. After a minimum of 1 week, fixed juveniles were carefully pried from the large shell on which they had settled and were decalcified in formic acid. After decalcification and rinsing, animals were processed in paraffin by standard methods (Humanson, 1979).

P. minimum cultures were diluted with 1G4F fixative (10 ml/100 ml culture) and concentrated by settling in an Imhoff cone. Supernatant was poured off, and concentrated cells were resuspended in 10 ml 1G4F for observation with transmission electron microscopy (TEM). These cultures were processed in paraffin with the agarose embedding methods described above.

Periodic acid-Schiff (PAS) staining of plastic-embedded sections was accomplished as follows: plastic was removed from thick sections of plastic-embedded larvae by incu-

Table I

Development of oyster embryos exposed for 48 h to cultured phytoplankton and culture extracts (means of three replicates, SD in parentheses)

Added	% Live normal	% Dead normal	% Live abnormal	% Dead abnormal
Seawater	91.3	0.4	5.9	2.4
	(1.5)	(0.4)	(0.9)	(0.6)
Medium	88.3	0.8	9.5	1.4
	(3.4)	(0.4)	(2.6)	(0.7)
Live T-ISO	89.5	1.0	8.4	1.2
	(2.8)	(0.8)	(2.5)	(0.2)
Live EXUV	84.1	1.7	9.3	4.9
	(4.0)	(0.8)	(1.3)	(2.2)
T-ISO Medium	88.9	0.7	9.3	1.1
	(1.3)	(0.4)	(1.0)	(1.0)
EXUV Medium	84.6	1.6	11.2	2.6
	(2.8)	(0.6)	(1.0)	(1.4)
Sonicated T-ISO	88.6	0.9	7.9	2.6
	(3.4)	(0.3)	(2.5)	(0.6)
Sonicated EXUV	80.2	1.5	15.2	3.1
	(1.0)	(0.8)	(2.4)	(2.2)
Heated T-ISO	74.2	1.0	24.1	0.7
	(25.7)	(0.4)	(25.2)	(0.1)
Heated EXUV	88.3	1.0	8.5	2.2
	(3.3)	(0.9)	(3.0)	(0.6)
Sonic, EXUV 5 ×	72.0	2.0	19.0	6.0
EXUV Medium 5×	87.0	2.0	10.0	1.0
Live EXUV 5×	88.0	1.0	9.0	1.0

bating slides with thick sections in a solution of 66% NaOH/33% ethanol for 15 min, followed by rehydration (Erlandsen *et al.*, 1979). Rehydrated sections were stained with Harris' hematoxylin counterstain (Sorvall, 1981) as previously described.

Results

Embryos

Counts of embryos developing to the straight-hinge larval stage after 48 h exposure to experimental treatments are shown in Table I. Development to straight-hinge stage was more than 80% in all treatments except for embryos exposed to heat-killed T-ISO and 5× sonicated EXUV; however, differences between these treatments and all others were not statistically significant (ANOVA, $p = 0.05$). Percentages of larvae in the other three categories were not statistically different either. Thus, none of the treatments interfered significantly with survival, development, or gross morphology as observed with the dissecting microscope.

Histologic observation by light and electron microscopy revealed only subtle differences between some treatments. Only samples of oysters exposed to seawater only, sonicated T-ISO, or 5× sonicated EXUV were examined. Although animals exposed to seawater only or to sonicated T-ISO had well-developed organ systems, oysters exposed to 5× sonicated EXUV showed mildly decreased numbers and sizes of lipid droplets in the mantle, velar cells, and absorptive cells of the digestive gland.

Feeding larvae

The salinity in all experimental cultures remained between 27‰ and 28‰ throughout the experiment, but differences in pH and *in vivo* fluorescence between treatments revealed differences in ingestion of the algal foods added and in the resultant water chemistry. Percentage of the diet consumed, averaged over the entire experiment, for each diet is listed in Table II. Larvae fed diets including EXUV filtered less of the diet from suspension. It was not, however, clear from fluorescence measurements alone if depressed filtration was a result of physical inability of the larvae to ingest *P. minimum* or to detrimental effects of the *P. minimum* cells that were ingested. The observation of *P. minimum* cells within digestive systems of larvae by epifluorescence microscopy and the histologic observations detailed below provided evidence that *P. minimum* cells were ingested. No significant differences in pH between diets were noted (Table II).

Differences in survival and growth of larvae on experimental diets were found, with *P. minimum* having consistent, detrimental effects. Numbers of larvae decreased for all feeding regimes, partly as a result of losses in sampling and handling. However, larvae fed only EXUV or the ⅔ EXUV + ⅓ T-ISO diet decreased in number more rapidly than larvae in the other treatments between days 13 and 17 (ANOVA, $p < 0.01$). Unfed larvae all starved within 27 days (Fig. 1). Growth of larvae on experimental diets was related directly to the amount of T-ISO in the diet (Fig. 2). Growth rates ranged from 1.10 to 6.52 μm d^{-1}, and differences between diets were significant (ANOVA, $p < 0.01$) (Table III).

Table II

Percentages of food filtered, as measured by in vivo *fluorescence decrease, and pH of larval culture water after 48-h incubation with larvae (means of 154 measurements, SE in parentheses)*

Feeding regime	% Fluorescence decrease	pH
T-ISO	36.1 (6.7)[1]	7.96 (0.02)[1]
⅓ EXUV + ⅔ T-ISO	32.4 (3.0)[1,2]	7.87 (0.05)[1]
⅔ EXUF + ⅓ T-ISO	14.9 (5.4)[2]	8.01 (0.01)[1]
EXUV	14.1 (8.6)[2]	7.94 (0.01)[1]
Unfed	−0.3 (0.3)[3]	7.89 (0.02)[1]

Different superscript numbers denote statistically different means by Duncan's multiple range test.

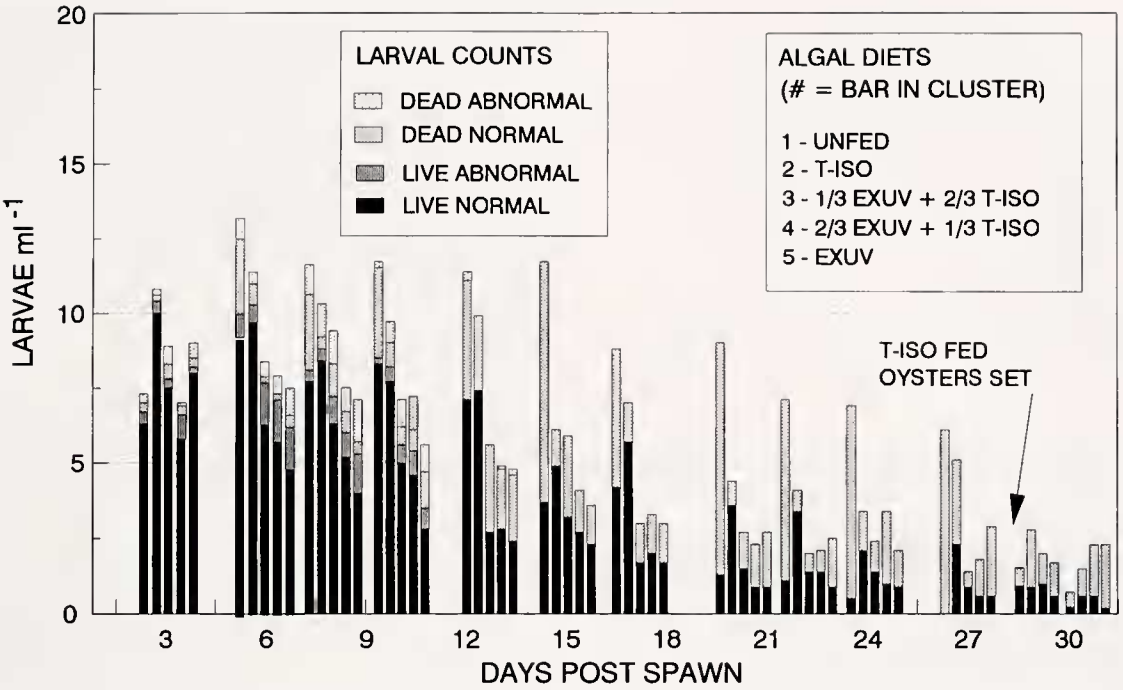

Figure 1. Counts of oyster larvae (means of three replicates) from experimental feeding regimes.

Histologic observation of larvae from day 8 revealed clear differences between EXUV-fed, T-ISO-fed, mixed-diet, and unfed treatments (Figs. 3, 4). Animals fed on T-ISO were in the prodissiconch II stage of development (Elston, 1980) and demonstrated excellent cellularity of developing organs (Fig. 3A). Stomach and intestinal epi-

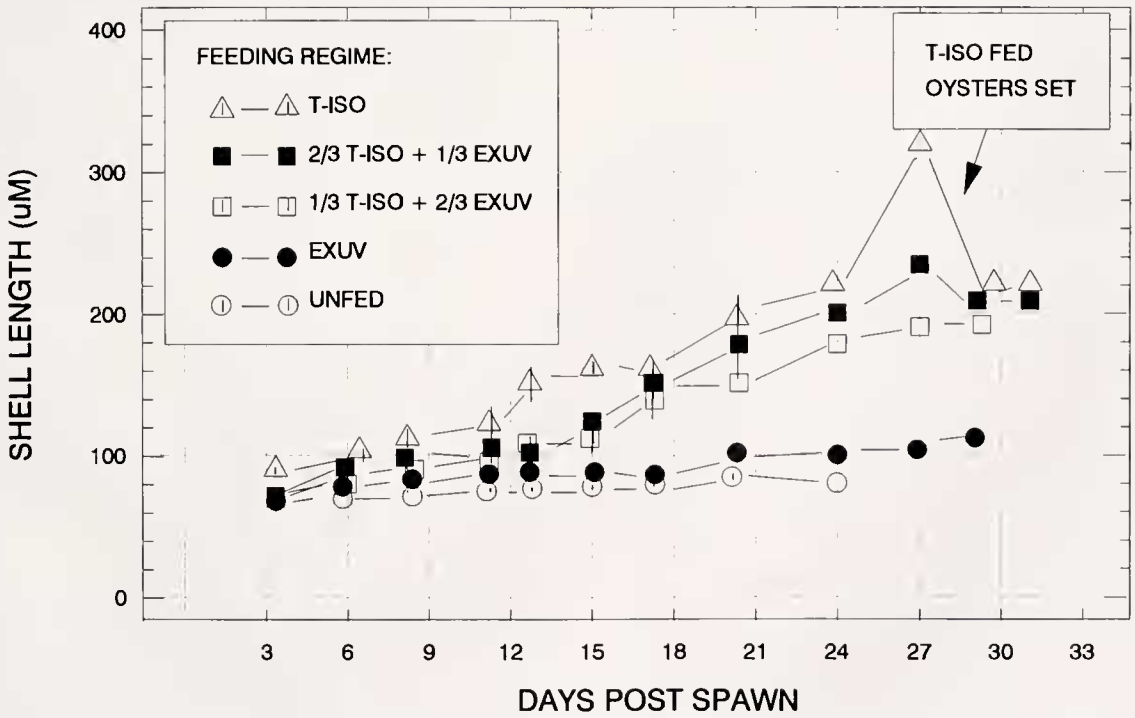

Figure 2. Shell-length measurements of larvae from experimental feeding regimes.

Table III

Growth rates, in micrometers per day (±SE), of larval oysters fed algal diets

Algal diet	Growth rate
Unfed	0.744 (.103)[a]
EXUV	1.10 (.447)[a]
⅔ EXUV	3.49 (.940)[ab]
⅓ EXUV	5.63 (1.33)[bc]
T-ISO	6.52 (1.54)[c]

Superscript letters indicate homogeneous subsets, according to Least Square Difference Multiple Range Test.

thelium was high-cuboidal in outline, and the intestinal epithelium contained many prominent lipid droplets. The digestive gland contained numerous tall-columnar absorptive cells. Examination with TEM showed that phagolysosomes within absorptive cell cytoplasm contained moderately electron-dense flocculent substances (Fig. 4A). Rare phagolysosomes present at the basal side of some cells contained dense, irregular inclusions.

Starved animals were still in the prodissiconch I stage of development (Elston, 1980). All organ development was poor, and organs demonstrated low cellularity (Fig. 3B). Stomach epithelium was low-cuboidal to squamous, and intestinal profiles were rare. Lipid droplets were not present in these animals. The digestive glands were small, with poor cellularity, as demonstrated by the greatly reduced numbers of low-columnar to cuboidal absorptive cells. Examination with TEM showed that rare phagolysosomes within the absorptive cell cytoplasm contained mildly flocculent contents.

All EXUV-fed larvae were in the late prodissiconch I stage of development. Organ development and cellularity was greater than in starved oysters, but was only about one-third that of T-ISO-fed animals (Fig. 3C). Stomach and intestinal epithelium was cuboidal to sometimes squamous. Lipid droplets were not seen in any cell type. Absorptive cells of the digestive glands were about one-third larger than in starved oysters. Interestingly, many dense inclusions were present throughout the cytoplasm of these cells (Fig. 3D). Examination with TEM showed these inclusions to be a continuum of phagolysosome profiles (which we have termed accumulation bodies)—from phagolysosomes containing possible autolysosome and other dinoflagellate debris, to those filled with densely flocculent material, to residual bodies containing haphazardly arranged, laminated, dense bundles of fibers with both loose and condensed appearances (Fig. 4B and 4C).

Eight-day-old oysters fed a diet containing ⅔ EXUV + ⅓ T-ISO were in the early prodissiconch II stage of development. Organ development and cellularity was slightly greater than in animals fed entirely on EXUV. Stomach and intestinal epithelia were cuboidal. Lipid droplets were rare in the stomach epithelium and very rare in the intestinal epithelium. Digestive glands appeared to be slightly more cellular than in animals fed EXUV only, as demonstrated by increased numbers of absorptive cells within any section of the glands. Phagolysosomes, as seen in control animals, and the distinctive accumulation bodies were noted under both light and transmission electron microscopy.

Newly set spat

Only larvae fed T-ISO alone or ⅓ EXUV + ⅔ T-ISO developed into pedi-veligers and set, and only about 100 spat were obtained with the latter diet including EXUV, as compared with more than 2000, from three initial populations of 2000, for the unialgal T-ISO diet. Spat from these treatments were maintained on their respective larval diets for 5 days after setting. Histologically, animals fed T-ISO alone exhibited well-developed organs with excellent cellularity (Fig. 5A). The stomach epithelium was cuboidal, with rare, small lipid granules in the cytoplasm. There were two to four profiles of intestine per section, and intestinal epithelial cells were cuboidal to columnar in outline. Lipid droplets were abundant and prominent in the intestinal epithelium. Phagocytes within the perivisceral cavity contained clear to lightly foamy cytoplasm. Absorptive cells of the digestive gland were of the tall-columnar type, with foamy, vacuolated cytoplasm. Examination with TEM demonstrated abundant phagolysosomes containing flocculent, irregular, granular material of various densities, as noted in pre-set larvae fed T-ISO.

Newly set spat fed the mixed diet showed distinctive and striking differences from the T-ISO controls (Fig. 5A). Animals contained moderately to poorly developed organ systems with reduced cellularity (Fig. 5B). Stomach epithelium was low-cuboidal to squamous and contained moderate to heavy accumulations of large lipid droplets. Only one to two cross-sections of intestine could be seen in some sections. Intestinal cells were cuboidal with variable (moderate to small) amounts of lipid droplets in the cytoplasm. Absorptive cells of the digestive gland were cuboidal to low-columnar in outline. Both light and electron microscopic examination showed that the absorptive cells contained abundant accumulation bodies, as described above (Fig. 5C). Examination of the intestinal epithelium with TEM showed that it also contained moderate numbers of accumulation bodies.

Distinctive effects of EXUV in the diet were seen in the perivisceral space of newly set oysters with both light and transmission electron microscopy. Phagocytes within the space were plump and although some contained only clear vacuoles, most phagocytes contained various numbers of large, dense, irregular particles, 3–

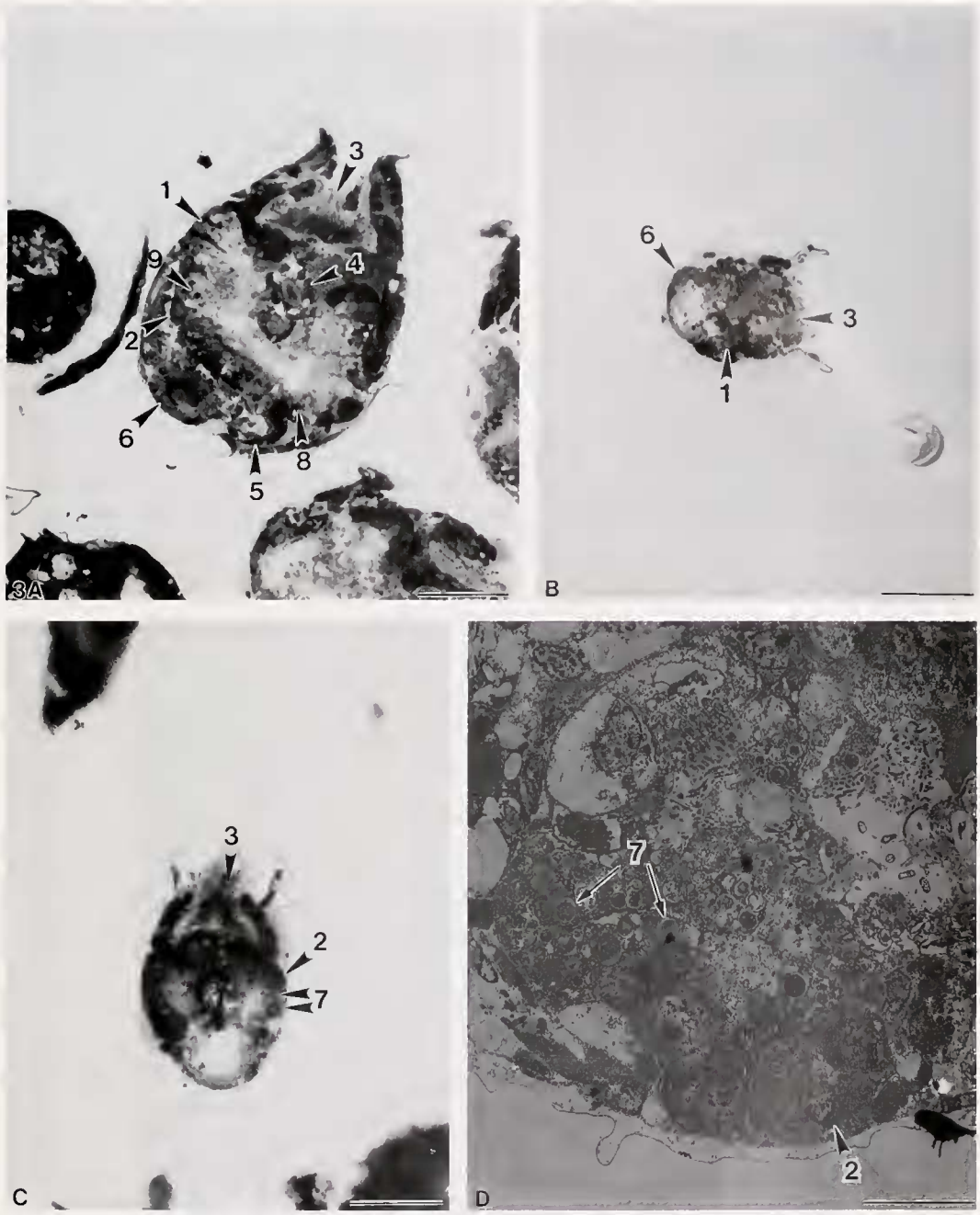

Figure 3. Feeding larvae. Photomicrographs A, B, and C are all at the same magnification. Animals are embedded in plastic, thick-sectioned, and stained with methylene blue (1, digestive gland absorptive cells; 2, digestive gland enzymatic cell; 3, velum; 4, esophagus; 5, intestine; 6, style sac; 7, accumulation bodies in absorptive cells; 8, lipid globules; 9, phagolysosomes). (A) Control animals (T-ISO-fed) show well-developed organs. (B) Starved larvae show poor development of all organs. (C and D) Animals fed 100% EXUV show poorly to moderately developed organs with distinctive accumulation bodies in the absorptive cells. (A to C, bar = 20 μm; D, bar = 12.5 μm.)

10 μm in diameter. Other phagocytes surrounded and appeared to be in the process of engulfing similar large, irregular, dense particles (Fig. 6A). Rarely, less degenerate particles showed profiles consistent with the dinoflagellate. Examination with TEM showed partially degraded dinoflagellates that contained small, round bodies (possible autolysosomes) (Fig. 6B). Focal, multicellular necrosis within organs was not evident in

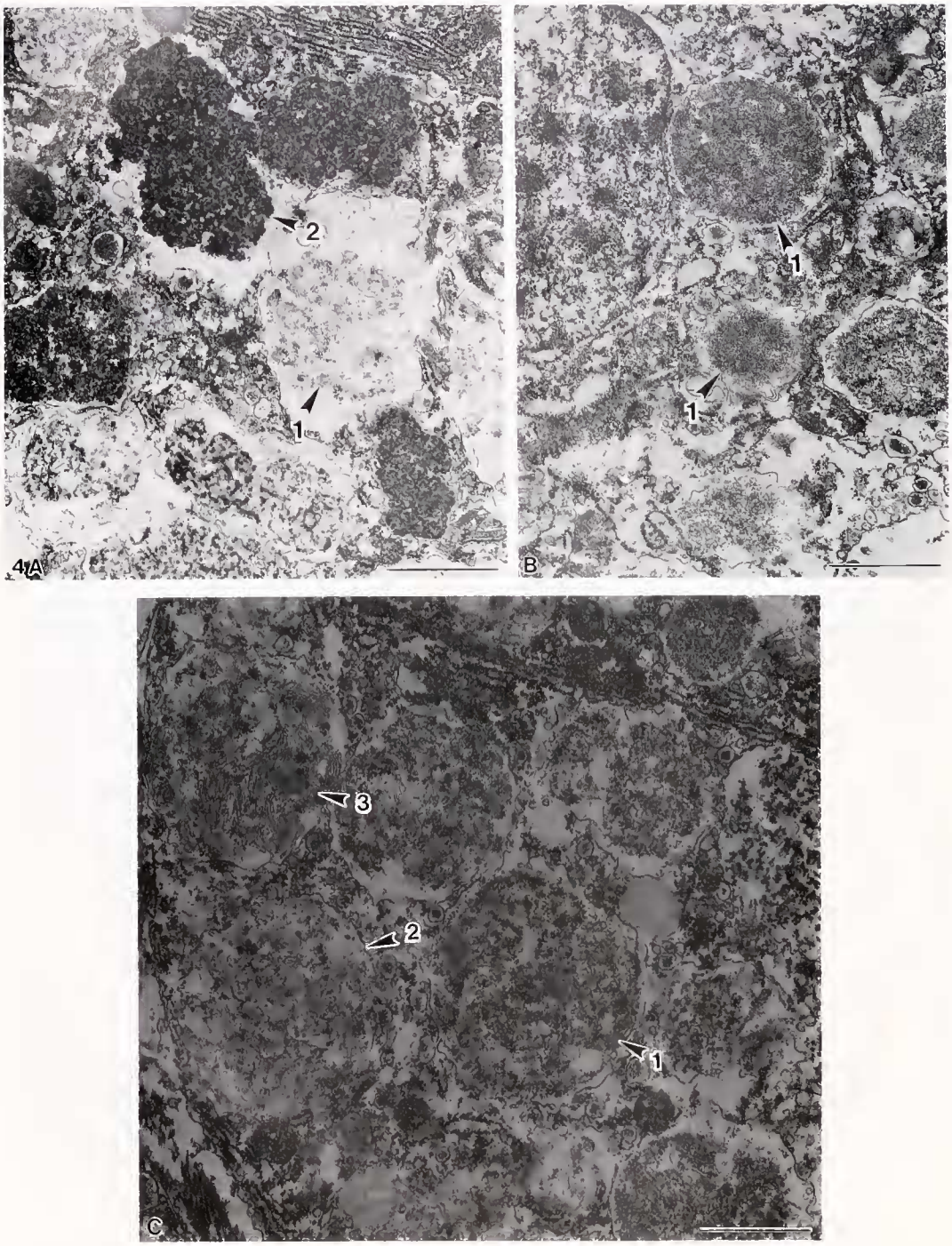

Figure 4. Feeding larvae (transmission electron micrographs). (A) T-ISO-fed animals show moderately electron-dense, flocculent substances (1) and dense, irregular inclusions (2) in phagolysosomes. (Bar = 2 μm.) (B and C) Distinctive accumulation bodies (oyster phagolysosomes containing dinoflagellate autolysosomes and other debris) are present in absorptive cells of EXUV-fed animals and show a progression from phagolysosomes containing debris and round bodies similar to autolysosomes (1), to phagolysosomes containing distinctive flocculent material (2), to residual bodies containing dense bundles of fibers (3). (B, bar = 2 μm; C, bar = 2 μm.)

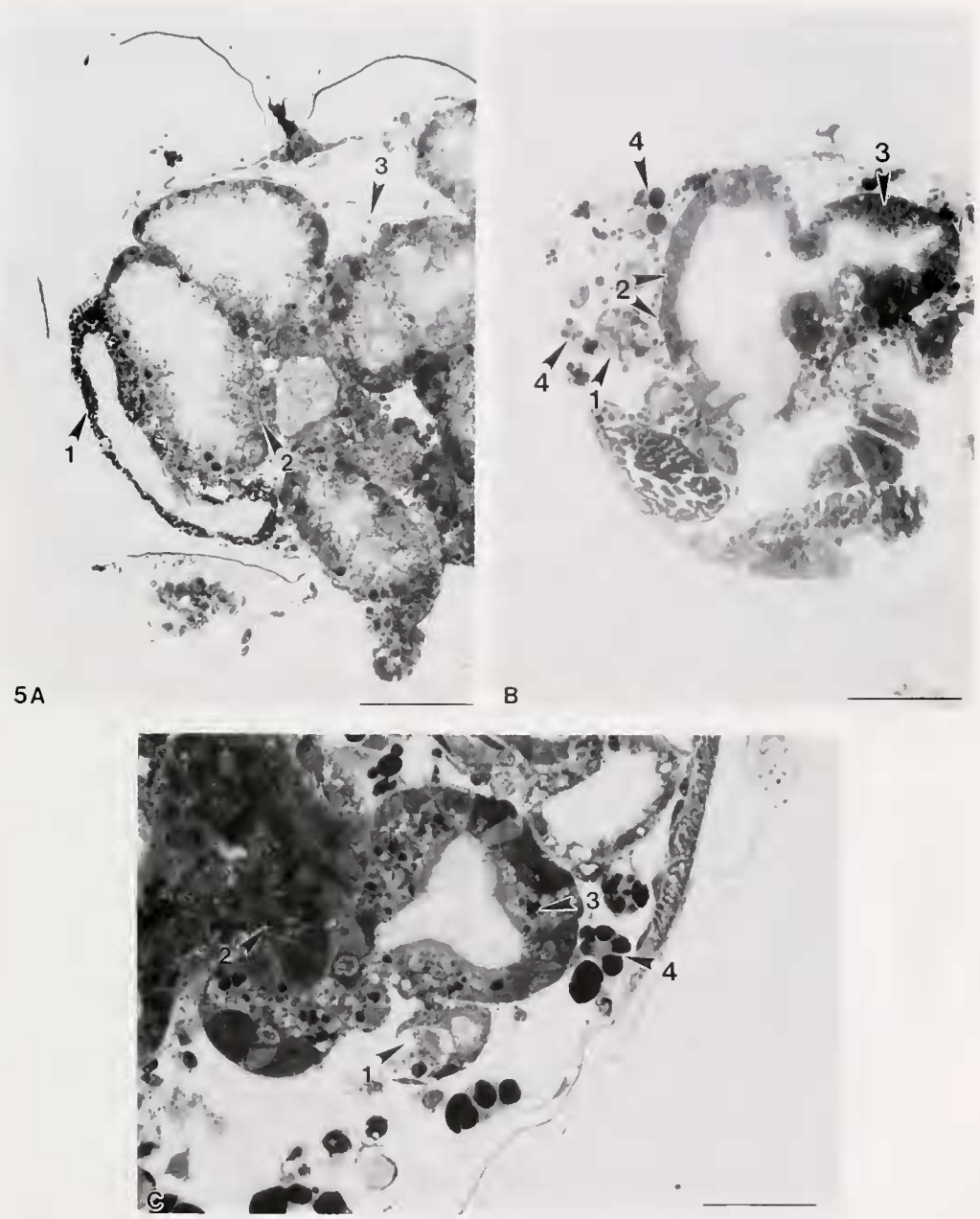

Figure 5. Newly set spat. Animals are embedded in plastic, thick-sectioned, and stained with methylene blue. (A) A T-ISO-fed control animal shows good development of organs with abundant lipid droplets in the intestinal epithelium (1), columnar absorptive cells in the digestive glands (2), and rare phagocytes in the perivisceral space (3). (Bar = 40 μm.) (B and C) An EXUV-fed animal shows poor development of intestine epithelium with vacuolation and lack of lipid droplets (1), abundant lipid droplets in the style-sac epithelium (2), and distinctive accumulation bodies in the absorptive cells of the digestive gland (3). Dinoflagellates in various stages of degeneration are present within phagocytes or are being engulfed by phagocytes in the perivisceral space (4). (B, bar = 40 μm; C, bar = 11.4 μm.)

EXUV-fed oysters; however, individual cell necrosis of the intestinal, digestive-gland, and stomach epithelium appeared to be present.

Histological examination of plastic-embedded larvae stained with PAS showed positive staining of accumulation bodies within the cytoplasm of absorptive cells of EXUV-fed (7A) but not T-ISO-fed oysters (Fig. 7B). The partially degraded dinoflagellates within the perivisceral space of newly set spat fed the mixed diet were positively stained with PAS.

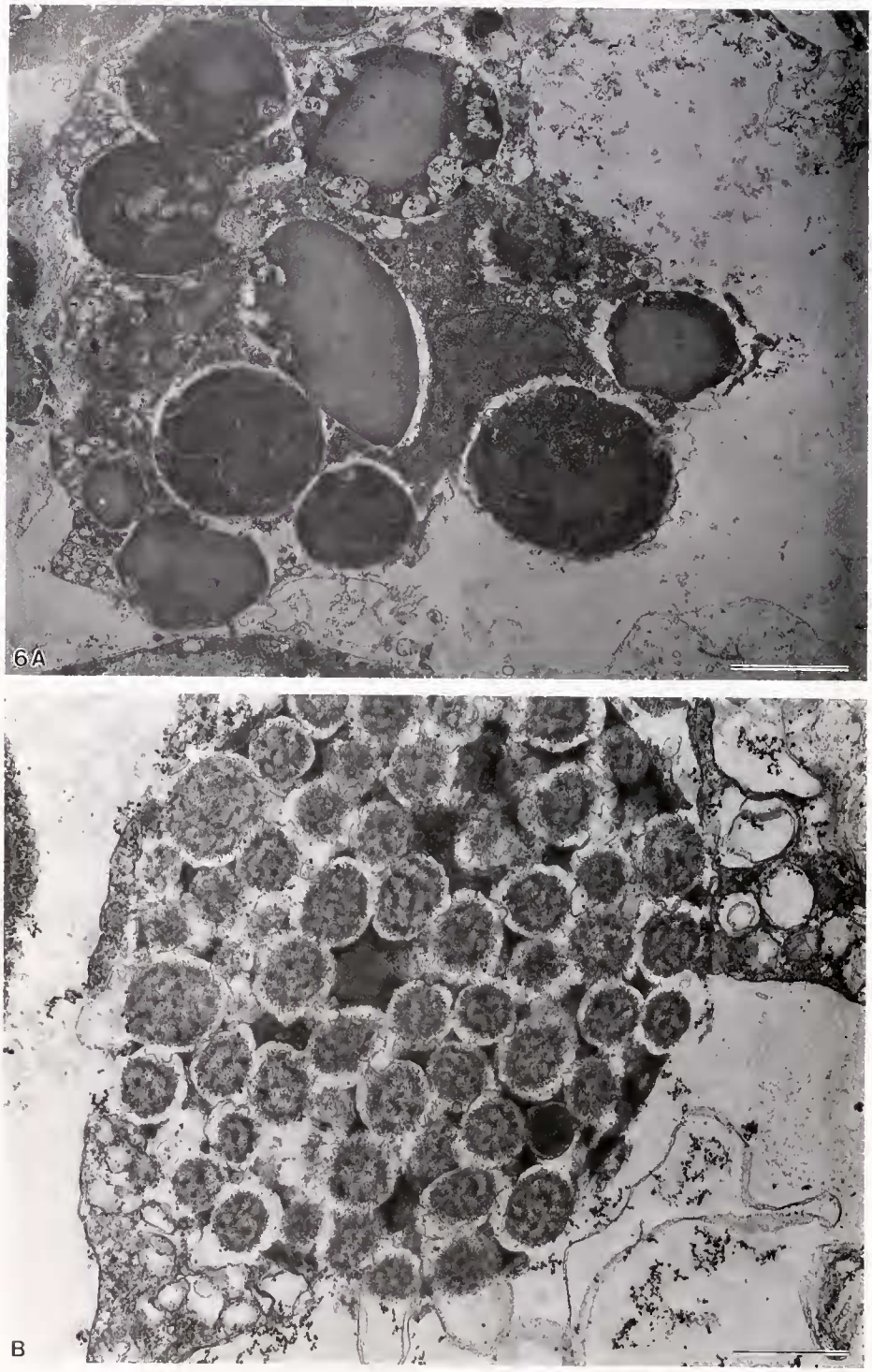

Figure 6. Newly set spat (transmission electron micrographs). (A) A perivisceral phagocyte of a mixed-diet-fed animal shows engulfment and digestion of the dinoflagellates. (Bar = 5 μm.) (B) A dinoflagellate within the perivisceral space has numerous autolysosomes. (Bar = 1.25 μm.)

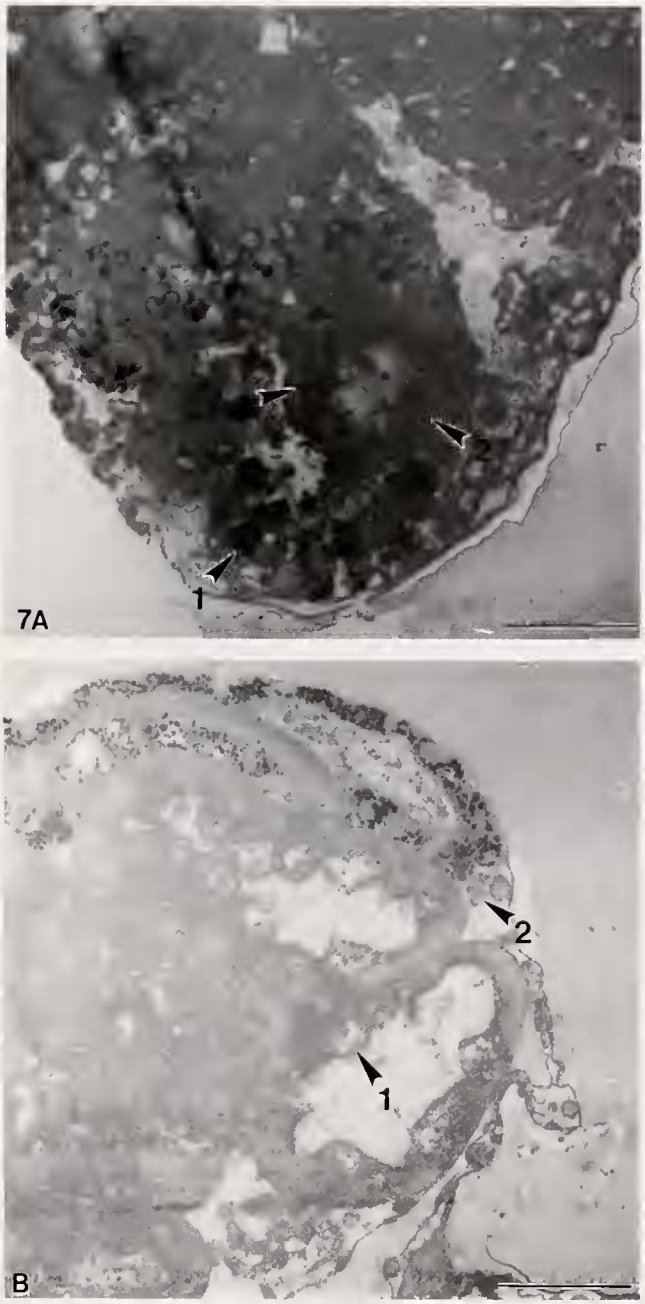

Figure 7. Newly set spat. PAS staining of plastic-embedded, thick-sectioned spat. (A) A mixed-diet-fed animal shows positive PAS staining of dinoflagellates in the perivisceral space (1) and accumulation bodies in the absorptive cells (2). (B) A T-ISO-fed animal shows no PAS staining in the perivisceral space (1) or in the absorptive cells of the digestive gland (2). Lipid granules are numerous in the intestine (3), and many absorptive cell nuclei are dense (4). (Bar = 40 μm.)

Juveniles

Juvenile oysters that had been feeding on natural Milford Harbor phytoplankton amended with cultured algae showed two different responses when subsequently exposed to experimental diets. Oysters fed T-ISO alone, or the ⅓ EXUV + ⅔ T-ISO diet, filtered the entire ration rapidly, produced normal fecal strands, and began growing immediately upon experimental feeding (Fig. 8). In contrast, oysters fed EXUV alone or the ⅔ EXUV + ⅓ T-ISO diet filtered the ration, but produced pseudofeces and grew poorly for the first week or two of experimental feeding. Thereafter, most oysters produced normal fecal strands and grew at rates the same as or higher than those on other diets (Table IV).

Histologic observations of oysters from each feeding regime sampled at the end of the experiment revealed that although the appearance of the absorptive cells in the digestive diverticula varied with diet, all were generally healthy except the negative controls (starved). Animals fed T-ISO showed absorptive cells with moderately columnar outlines and consistent foamy, vacuolated cytoplasm (Fig. 9A). In addition to generally poorly developed organ systems, starved oysters showed digestive diverticula cells that were cuboidal to low-columnar in outline with vacuolated/fine granular cytoplasm (Fig. 9B). In general, animals fed partially or wholly on EXUV had abundant columnar epithelia with taller absorptive-cell outlines than those seen in T-ISO-fed animals. The cytoplasmic contents of EXUV-fed animals were similar to those of the positive (T-ISO-fed) controls in vacuolation, but were more coarsely granular (Fig. 9C); additionally, reserve cells appeared to be more common in these diverticula. Interestingly, a subset of several oysters from the EXUV unialgal feeding regime exhibited varying degrees of development of absorptive cells. In the most severely affected oysters from this subset, the digestive gland epithelial cells were cuboidal to low-columnar, with relatively homogeneous eosinophilic cytoplasm intermingled with many brown granules about 2 μm in size (Fig. 9D).

Discussion

Our previous experience with feeding the EXUV strain of *P. minimum* to other molluscs led us to expect that oysters could experience detrimental effects that might include mortality, as with scallops, or growth inhibition, as with clams (Wikfors and Smolowitz, 1993). Exposures of prefeeding oyster embryos to spent algal growth media and live, killed, and extracted cells were intended to determine if a soluble toxin would affect embryonic development; if so, larval bioassay might be a useful method for quantification of toxin content in *P. minimum*. This clearly is not the case. No experimental treatments yielded statistically significant differences in development, and any possible histological effects were subtle and fell within the limits of normal variation. Lack of detrimental effects upon prefeeding larvae could, however, have been attributed to insufficient dosage in the experimental protocol, although even the higher, 5× EXUV exposures were not

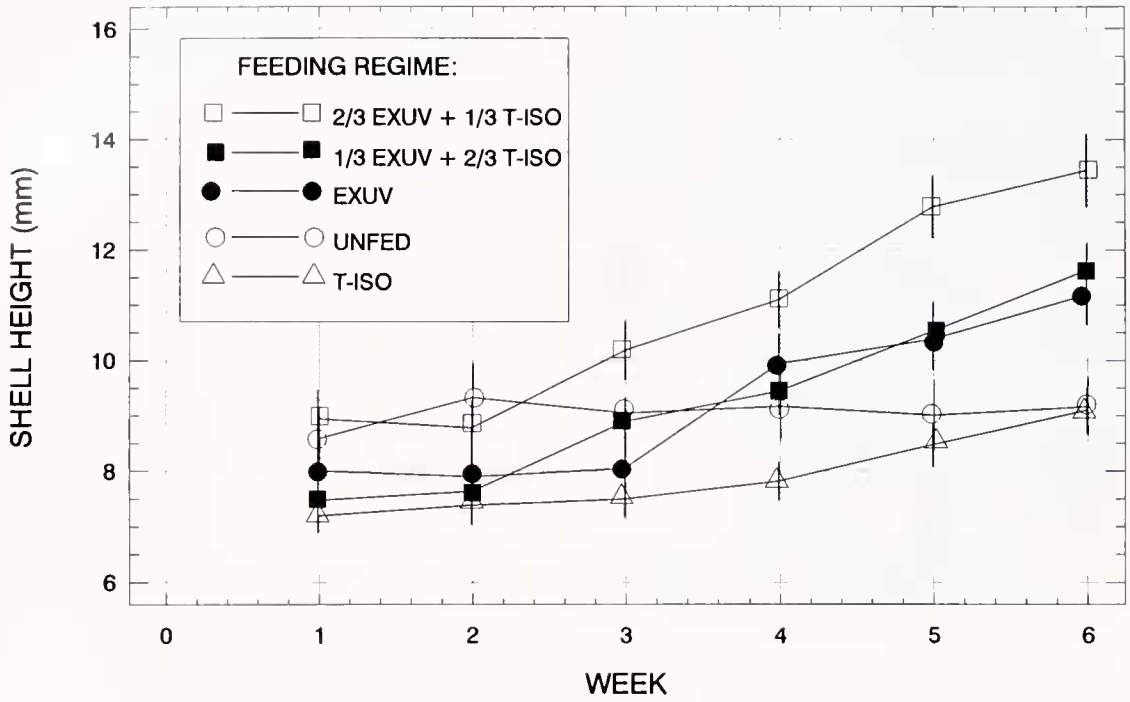

Figure 8. Shell-length measurements of juvenile oysters from experimental feeding regimes.

harmful (Table I). Because our experimental exposures were greater than exposures expected in natural waters, except possibly under extreme bloom conditions, we conclude that harm to oyster embryos from *P. minimum* exudates is unlikely in nature. Furthermore, it appears unlikely that the putative molluscan enterotoxin in this dinoflagellate is released into solution in a form harmful to shellfish. Davis and Chanley (1956) reported that a dinoflagellate bloom in Milford Harbor (most likely a species of *Prorocentrum*) interfered with development of oyster and clam embryos in filtered seawater; however, the effectiveness of the filtration in removing the dinoflagellate cells was not evaluated. The "bag-filtering" process employed at Milford at that time probably would not remove *P. minimum* effectively.

Feeding *P. minimum* to oyster larvae yielded clear detrimental effects, but it was not apparent from experimental results alone if reduced survival and growth resulted from toxic effects or from starvation. EXUV, with a size of about 9×6 μm, is near the maximum size of particles that molluscan larvae can ingest (Riisgard *et al.*, 1980). Observations of fed larvae by epifluorescence microscopy, and monitoring of larval-culture fluorescence after feeding, indicated that larval consumption was lower with EXUV than with T-ISO (Table II). Epifluorescence microscopy and histologic evidence do, however, support the contention that larvae can ingest EXUV cells. Accumulation bodies originating from intracellular digestion of EXUV cells were seen in larvae fed EXUV, but not in larvae starved or fed T-ISO alone. Thus, pathologic

Table IV

Growth rates, in millimeters per week, of juvenile oysters fed algal diets

	Diet				
Week interval	Unfed	EXUV	⅔ EXUV	⅓ EXUV	T-ISO
1–2	0.74 (.067)[a]	−0.10 (.010)[c]	−0.16 (.023)[c]	0.16 (.027)[b]	0.19 (.047)[b]
2–3	−0.28 (.056)[c]	0.15 (.015)[b]	1.4 (.036)[a]	1.3 (.039)[a]	0.11 (.019)[b]
3–4	0.12 (.056)[c]	1.9 (.039)[a]	0.91 (.022)[b]	0.55 (.017)[c]	0.32 (.018)[d]
4–5	−0.16 (.012)[d]	0.44 (.025)[c]	1.7 (.056)[a]	1.2 (.077)[b]	0.66 (.047)[c]
5–6	0.15 (.011)[c]	0.80 (.021)[b]	0.66 (.093)[b]	0.98 (.043)[a]	0.62 (.020)[b]

Superscript letters indicate homogeneous subsets, according to Least Square Difference Multiple Range Test.

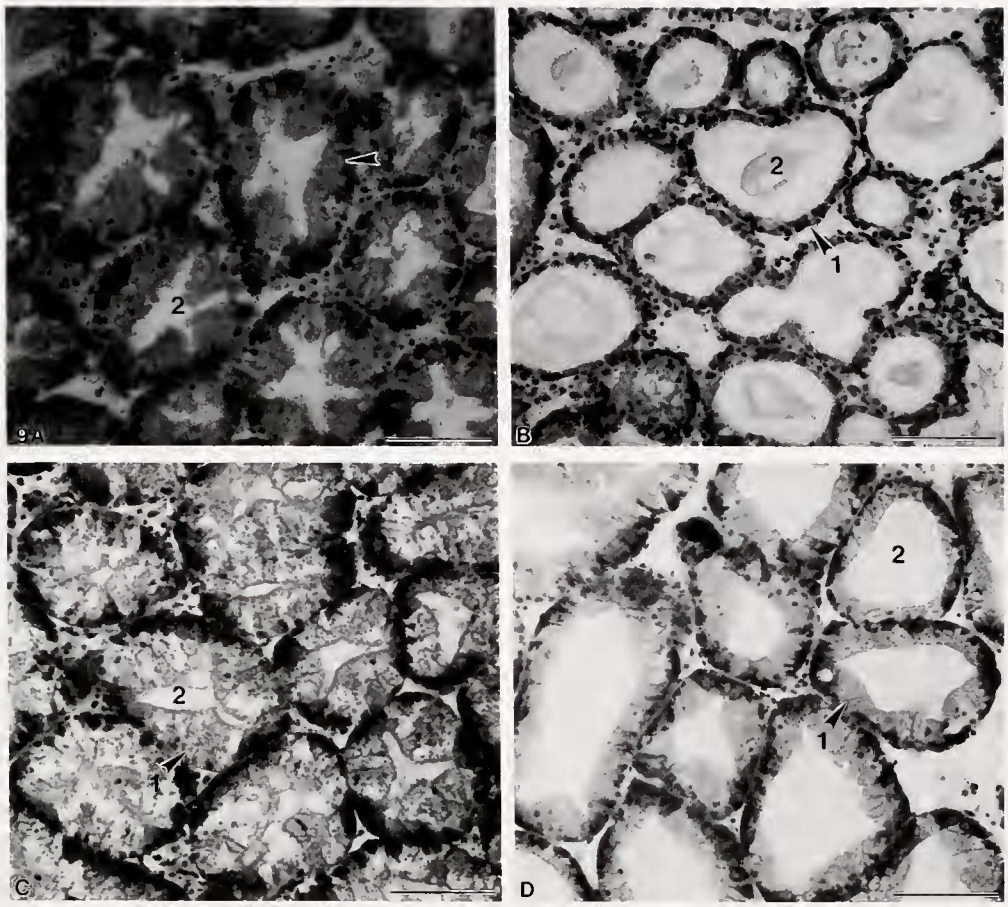

Figure 9. Juvenile oysters. Sections of paraffin-embedded animals are stained with hematoxylin and eosin. (Bar = 40 μm.) (A) A positive control animal (fed 100% T-ISO) shows moderate columnar absorptive cells (1) and star-shaped lumens (2). (B) Starved juveniles show cuboidal epithelium (1) with dilated, rounded lumens (2). (C) Most EXUV-fed animals showed high-columnar epithelium with foamy cytoplasm (1) and small lumens (2). (D) Some EXUV-fed animals showed low-columnar epithelium (1) and dilated lumens (2).

changes associated specifically with *P. minimum* feeding were identified; the chief feature was the presence of accumulation bodies within absorptive cells of the digestive diverticula. Metamorphosis and setting of very few larvae fed ⅓ EXUV + ⅔ T-ISO (less than 100, as compared with more than 2000 in larvae reared on T-ISO alone) provided further evidence that limited growth and development in the mixed algal diet was not simply a result of quantitative malnutrition.

The presence of accumulation bodies within absorptive cells of digestive diverticula in larvae fed 100% EXUV suggests that some specific component of the *P. minimum* cell interfered with cellular digestive processes. Similar accumulation bodies were found in recently set spat that we continued to feed the larval diet of ⅓ EXUV + ⅔ T-ISO. These spat also had large amounts of lipid in the stomach, but modest to very low amounts of lipid in the moderately to poorly developed intestinal epithelium. In contrast, oysters fed T-ISO alone had large amounts of lipid in the moderately to well-developed intestine and only rare lipid droplets in the stomach. Clearly, digestive processes in larvae and young spat were disrupted by the presence of *P. minimum* in the diet.

The occurrence of large numbers of partially digested dinoflagellates within phagocytes of the perivisceral cavity again demonstrated the reduced ability of the oysters' cells to degrade these organisms even though they did elicit a phagocytic response. Only rarely, however, was a relatively intact dinoflagellate seen in the perivisceral space, and even those were in the process of being, or had just been, phagocytized. We do not know how these organisms, either whole or partially degraded, entered the perivisceral space, but they could have passed through breaks in the epithelial integrity of the digestive gland or intestine during setting. Certainly their occurrence within the perivisceral space would significantly stress the animals.

In light of results with younger animals, findings with juvenile oysters were somewhat surprising. A previous study on filtration of EXUV by adult *C. virginica* revealed that cultured EXUV was filtered from suspension but not digested very effectively in short-term exposures (Shumway *et al.*, 1985). Further experiments by Luckenbach *et al.* (1993) demonstrated complete mortality in 14 days of juvenile oysters fed a high density of *P. minimum* (a Chesapeake Bay isolate). Oysters fed a lower density of this same culture showed 43% mortality in the first 22 days of exposure, after which survival stabilized; 5% *P. minimum* added to a mixed algal diet improved growth of oysters in these latter experiments (Luckenbach *et al.*, 1993). Thus, in the present study the initial depression in growth rates of oysters fed EXUV alone—or as a mixed diet of ⅔ EXUV and ⅓ T-ISO—was expected. The sudden acceleration in growth on these diets after 2 weeks was different from results obtained by Luckenbach *et al.* (1993), but it was not wholly inconsistent with their findings. The survival of all oysters still alive after 22 days of exposure to the lower *P. minimum* concentration in the Luckenbach *et al.* (1993) study suggests that some oysters are more resistant than others, or that some recovery is possible. Our feeding regime resulted in feeding densities within the "bloom" (high) range of Luckenbach *et al.* (1993); hence, differences in mortality of oysters in the two studies may result from differences in the algal strains or in the initial nutritional condition of the oysters. In our experiment, before oysters were used in exposure trials they were fed a diet known to support rapid growth; they may have had sufficient nutrient stores to survive initial stress.

Growth data from the present study show widening variance as mean sizes increased, suggesting that some oysters were growing more rapidly than others. A number of oysters were selected, therefore, for histological examination. Individuals that were from the EXUV-fed group and had well-developed absorptive cells were most likely growing rapidly. In contrast, some oysters from the same group were in poor condition, judging by digestive-system appearance, and resembled younger oysters fed *P. minimum* in their histologic characteristics, including low-columnar epithelium and comparatively large, granular cytoplasmic inclusions. The intragroup variance in the appearance of the digestive diverticula cells is interesting. We speculate that spat needed specific enzymes to digest or eliminate *P. minimum* material that might otherwise accumulate in epithelial cells of the digestive diverticula. Whether such enzymes are induced by the presence of the dinoflagellate or occur as part of the normal developmental sequence of the digestive gland is unknown. In the feeding larvae and newly set spat, we believe that the contents of accumulation bodies, when present in large amounts within the absorptive cells, may have interfered with digestion.

The nature of the material contained within accumulation bodies in absorptive cells was investigated through PAS staining of oyster tissues. We were fortunate to obtain sections through *P. minimum* cells inside the digestive lumen of the oyster. These sections revealed structures within the dinoflagellate cells that appeared in TEM preparations to correspond with dinoflagellate autolysosomes (Schmitter, 1971; Schmitter and Jurkiewicz, 1981). About 20 of these organelles were seen within each dinoflagellate. A recent report has demonstrated that autolysosomes in *Prorocentrum* cells are strongly PAS positive (Zhou and Fritz, 1994a); individual *Prorocentrum* cells contained more than 20 autolysosomes, corresponding well with our observations. PAS reaction offered a possible method for investigating whether accumulation bodies in oyster absorptive cells contained material from *P. minimum* autolysosomes. PAS-positive staining of accumulation bodies in absorptive cells of oysters fed *P. minimum*, but not in oysters fed T-ISO, is strong evidence that dinoflagellate autolysosomes are involved in the detrimental effects that some dinoflagellates have upon shellfish. The evidence is not definitive: some substance produced by the oyster lysosome could be responsible for the PAS staining seen in newly set spat. We have, however, no evidence that this is the case. Widdows *et al.* (1979) noted a similar increase in PAS-positive phagolysosomes of pathologically affected absorptive cells in *Mytilus edulis* fed *Gyrodinium aureolum*.

Comparing results of this study with our previous laboratory exposures of northern quahogs and bay scallops to *P. minimum* (Wikfors and Smolowitz, 1993) suggests a possible scenario linking dinoflagellate autolysosomes with effects upon shellfish. Quahogs did not grow during six weeks of feeding on diets containing EXUV, but no mortalities occurred; apparently these bivalves were unable to digest EXUV. Unfortunately, we did not examine these clams histologically to determine if poor digestion resulted from damage to digestive diverticula or from inability of enzymes in the digestive lumen to lyse dinoflagellate cells. In bay scallops fed a diet containing EXUV, mortality was rapid. No accumulation bodies were seen in scallop absorptive cells; instead, these cells appeared to be rapidly sloughed into the lumen. If digestive enzymes in the scallops' diverticular lumen were able to lyse *P. minimum* cells and autolysosomes, thereby releasing dinoflagellate autolysosomal enzymes into the lumen, and if these dinoflagellate enzymes were damaging to scallop absorptive cells, then the enterotoxic effects we observed could have resulted. Bay scallops are thought to accomplish essentially all digestion extracellularly within the digestive-gland lumen (Reid, 1982); oysters, on the other hand, are considered to be phagotrophs (Galtsoff, 1964).

In oysters, dinoflagellate autolysosomes may be taken whole by phagocytosis into absorptive cells, where partial degradation and subsequent accumulation as persistent phagolysosome (residual bodies) occurs. We have termed these characteristic phagolysosomes and residual bodies "accumulation bodies." Older, juvenile oysters appear to develop digestive enzymes, either released into the lumen or within absorptive cells, that completely digest the dinoflagellates—including any dinoflagellate-derived autolysosomes. It is interesting to note that feeding algal cells contaminated with cadmium to juvenile oysters causes degradation of the diverticular epithelium that is similar to the effects of *P. minimum* upon bay scallops (Wikfors, in prep.). Apparently *P. minimum* does not release a chemical toxin into the diverticular lumen of the oyster, though it appears to do so in the scallop. A recent study has shown that okadaic acid (DSP toxin) is localized in organelles, including autolysosomes, in *P. lima* and *P. maculosum* (Zhou and Fritz, 1994b); thus, digestive degradation of the organelles seems to be necessary for release of the toxin. Differences in symptoms in different bivalves seem to offer clues as to how the molluscs respond to *P. minimum*, in terms of digestive sequences and sites, and to highlight the diversity of digestive methods and enzymes among various types of bivalves. Further research is needed to elucidate fully the possible relationships between dinoflagellate autolysosomes, molluscan digestive processes, and susceptibility of bivalve molluscs to harmful algal blooms.

Acknowledgments

This study was supported by the NOAA Oyster Disease Initiative, administered through the Northeast Region of NOAA's National Marine Fisheries Service.

Technical assistance of B. C. Smith, L. Garvey, T. Godfrey, and Michelle McCafferty is gratefully acknowledged. We thank S. E. Shumway for providing bioassay results of cultured *P. minimum*. Constructive comments were provided by E. N. Wikfors, R. A. Robohm, and A. Calabrese.

Literature Cited

Babinchak, J., and R. Ukeles. 1979. Epifluorescence microscopy, a technique for the study of feeding in *Crassostrea virginica* veliger larvae. *Mar. Biol.* **51:** 69–76.

Davis, H. C., and P. E. Chanley. 1956. Effects of some dissolved substances on bivalve larvae. *Proc. Natl. Shellfish. Assoc.* **47:** 59–74.

Dodge, J. D. 1982. *Marine Dinoflagellates of the British Isles.* Her Majesty's Stationery Office, London.

Elston, R. 1980. *The Larval American Oyster,* Crassostrea virginica. *Its Functional Anatomy, Histology and Ultrastructure and Response to Challenge with the Bacterium, Vibrio spp.* Cornell University, Ph.D. Thesis.

Erlandsen, S. L., J. A. Parsons, and C. B. Rodning. 1979. Technical parameters of immunostaining of osmicated tissue in epoxy sections. *J. Histochem. Biochem.* **27:** 1286–1289.

Gainey, L. F., and S. E. Shumway. 1988. A compendium of the responses of bivalve mollusks to toxic dinoflagellates. *J. Shellfish Res.* **7:** 623–628.

Galtsoff, P. S. 1964. The American oyster. *Fishery Bull.* **64:** 1–480.

Hallegraeff, G. M. 1993. A review of harmful algal blooms and their apparent global increase. *Phycologia* **32:** 79–99.

Howard, D. W., and C. S. Smith. 1983. *Histological Techniques for Marine Bivalve Mollusks.* NOAA Technical Memorandum NMFS-F/NEC-25. U.S. Dept. of Commerce.

Humanson, G. L. 1979. Pp. 210–212 in *Animal Tissue Techniques.* W. H. Freeman and Co., San Francisco.

Loosanoff, V. L., and H. C. Davis. 1963. Rearing of bivalve mollusks. *Adv. Mar. Biol.* **1:** 1–136.

Luckenbach, M. W., K. G. Sellner, S. E. Shumway, and K. Greene. 1993. Effects of two bloom-forming dinoflagellates, *Prorocentrum minimum* and *Gyrodinium uncatenum,* on the growth and survival of the eastern oyster, *Crassostrea virginica* (GMELIN 1791). *J. Shellfish Res.* **12:** 411–415.

Marshall, H. G. 1980. Seasonal phytoplankton composition in the lower Chesapeake Bay and Old Plantation Creek, Cape Charles, Virginia. *Estuaries* **3:** 207–216.

Parry, G. D., J. S. Langdon, and J. M. Huisman. 1989. Toxic effects of a bloom of the diatom *Rhizosolenia chunii* on shellfish in Port Phillip Bay, Southeastern Australia. *Mar. Biol.* **102:** 25–41.

Reid, R. G. B. 1982. Aspects of bivalve feeding and digestion relevant to aquaculture nutrition. Pp. 231–251 in *Proceedings of the Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition.* G. D. Pruder, C. J. Langdon, and D. E. Conklin, eds. Louisiana State University, Baton Rouge.

Rhodes, E. W., and W. S. Landers. 1973. Growth of oyster larvae, *Crassostrea virginica,* of various sizes in different concentrations of the chrysophyte, *Isochrysis galbana. Proc. Natl. Shellfish. Assoc.* **63:** 53–59.

Riisgard, H. U., A. Randlov, and P. S. Kristensen. 1980. Rates of water processing, oxygen consumption and efficiency of particle retention in veligers and young post-metamorphic *Mytilus edulis. Ophelia* **19:** 37–47.

Sakamoto, Y., R. F. Lockey, and J. J. Krzanowski, Jr. 1987. Shellfish and fish poisoning related to the toxic dinoflagellates. *Southern Med. J.* **80:** 866–872.

Schmidt, R. J., and A. R. Loeblich, III. 1979. Distribution of paralytic shellfish poison among Pyrrophyta. *J. Mar. Biol. Assoc. U. K.* **59:** 479–487.

Schmitter, R. E. 1971. The fine structure of *Gonyaulax polyedra,* a bioluminescent marine dinoflagellate. *J. Cell Sci.* **9:** 147–173.

Schmitter, R. E., and A. J. Jurkiewicz. 1981. Acid phosphatase localization in PAS bodies of *Gonyaulax. J. Cell Sci.* **51:** 15–23.

Sellner, K. G., F. Sawangwong, R. Dawson, W. R. Boynton, W. M. Kemp, and J. H. Garber. 1993. Fate of dinoflagellates in Chesapeake Bay: Is sedimentation likely? Pp. 825–830 in *Toxic Phytoplankton Blooms in the Sea.* T. J. Smayda and Y. Shimizu, eds. Elsevier, Amsterdam.

Shumway, S. 1990. A review of the effects of algal blooms on shellfish and aquaculture. *J. World Aquacult. Soc.* **21:** 65–104.

Shumway, S. E., T. L. Cucci, R. C. Newell, and C. M. Yentsch. 1985. Particle selection, ingestion, and absorption in filter-feeding bivalves. *J. Exp. Mar. Biol. Ecol.* **91:** 77–92.

Sorvall Instruction Manual. 1981. *Staining Procedures for Plastic Embedded Tissue.* DuPont Company, Instrument Products, Biomedical Division, Newtown, Connecticut.

Steidinger, K. A. 1983. A re-evaluation of toxic dinoflagellate biology and ecology. Pp. 148–188 in *Progress in Phycological Research.* F. E. Round and D. J. Chapman, eds. Elsevier, New York.

Ukeles, R. 1973. Continuous culture—a method for the production of unicellular foods. Pp. 233–254 in *Handbook of Phycological*

Methods—Culture Methods and Growth Measurements. J. R. Stein, ed. Cambridge University Press, London.

Ukeles, R., and G. H. Wikfors. 1982. Design, construction, and operation of a rearing chamber for spat of *Crassostrea virginica* (Gmelin). *J. Shellfish Res.* **2:** 35–39.

Wikfors, G. H., and R. M. Smolowitz. 1993. Detrimental effects of a *Prorocentrum* isolate upon hard clams and bay scallops in laboratory feeding studies. Pp. 447–452 in *Toxic Phytoplankton Blooms in the Sea.* T. J. Smayda and Y. Shimizu, eds. Elsevier, Amsterdam.

Widdows, J., M. N. Moore, D. M. Lowe, and P. N. Salkeld. 1979. Some effects of a dinoflagellate bloom (*Gyrodinium aureolum*) on the mussel, *Mytilus edulis. J. Mar. Biol. Assoc. U. K.* **59:** 522–524.

Zhou, J., and L. Fritz. 1994a. PAS/accumulation bodies in *Prorocentrum lima* and *Prorocentrum maculosum* (Dinophyceae) are dinoflagellate autolysosomes. *J. Phycol.* **30:** 39–44.

Zhou, J., and L. Fritz. 1994b. (abstract). DSP toxin localizes to plastids in producer *Prorocentrum* cells. ASLO & PSA 1994 Joint Meeting Conference Program and Book of Abstracts, p. a-85.